

Modulation of Type I interferon-associated viral sensing during acute simian immunodeficiency virus (SIV) infection in African green monkeys

Running title: Very early effects of SIV infection on viral sensing

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21 **Abstract**

22 Natural hosts of simian immunodeficiency virus (SIV), such as African green monkeys (AGMs),
23 do not progress to AIDS. This associates with an absence of a chronic type I interferon (IFN-I)
24 signature. It is unclear how the IFN-I response is downmodulated in AGMs. We longitudinally
25 assessed the capacity of AGM blood cells to produce IFN-I in response to SIV and Herpes
26 Simplex virus (HSV). Phenotype and function of plasmacytoid dendritic cells (pDCs) and other
27 mononuclear blood cells were assessed by flow cytometry and expression of viral sensors
28 measured by RT-PCR. pDC displayed low BDCA-2, CD40 and HLA-DR expression during acute
29 SIVagm infection. BDCA-2 was required for sensing SIV, but not HSV, by pDCs. In acute infection,
30 AGM PBMCs produced less IFN-I upon SIV stimulation. In chronic phase the production was
31 normal, confirming that the lack of chronic inflammation is not due to a sensing defect of pDCs.
32 In contrast to stimulation by SIV, more IFN-I was produced upon HSV stimulation of PBMC
33 isolated during acute infection, while the frequency of AGM pDCs producing IFN-I upon in vitro
34 stimulation with HSV was diminished. Indeed, other cells started producing IFN-I. This increased
35 viral sensing by non-pDCs was associated with an upregulation of TLR3 and IFI16 caused by IFN-I
36 in acute SIVagm infection. Our results suggest that, as in pathogenic SIVmac infection, SIVagm
37 infection mobilizes bone marrow precursor pDC. Moreover, we show that SIV infection modifies
38 the capacity of viral sensing in cells other than pDCs which could drive IFN-I production in
39 specific settings.

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41

42 **Importance**

43 The effect of HIV/SIV infections on the capacity of plasmacytoid dendritic cells (pDC) to produce
44 IFN-I in vivo is still incompletely defined. As IFN-I can restrict viral replication, contribute to
45 inflammation and influence immune responses, alteration of this capacity could impact viral
46 reservoir size. We observed that even in non-pathogenic SIV infection, the frequency of pDC
47 capable to efficiently sense SIV and produce IFN-I is reduced during acute infection. We
48 discovered that, concomitantly, cells other than pDCs increased their ability of viral sensing. Our
49 results suggest that pDC-produced IFN-I upregulate viral sensors in bystander cells, the latter
50 gaining the capacity to produce IFN-I. These results indicate that in certain settings, cells other
51 than pDCs can drive IFN-I-associated inflammation in SIV infection. This has important
52 implications for the understanding of persistent inflammation and the development of anti-
53 inflammatory treatment strategies in HIV infection.

54 **Introduction**

55 Type I Interferons (IFN-I) play an important role in Human immunodeficiency virus (HIV) and
56 simian immunodeficiency virus (SIV) infection in humans and macaques (MACs). IFN-I can limit
57 viral replication through several mechanisms: upregulation of restriction factors (23),
58 maturation of antigen-presenting cells (13), activation of NK cells (42) and induction of
59 apoptosis in infected cells (22). Indeed, exogenous administration of IFN α in chronically infected
60 HIV patients decreased viremia by seventy-five percent (2). Similarly, IFN α administration in the
61 chronic phase of SIVagm or SIVsmm infection reduced viral loads (20, 50). In one study, it could

62 suppress viral rebound in HIV patients upon antiretroviral treatment interruption (3). Finally,
63 IFN-I blockage in acute SIVmac infection increased the viral reservoir and accelerated
64 progression to AIDS (45). However, IFN-I production can also be detrimental: IFN-I can
65 contribute to chronic immune activation (IA) (18), induce apoptosis of uninfected bystander
66 cells (17) and drive cells to a short-lived effector cell differentiation program (46). Interferon-
67 stimulated gene (ISG) expression associates with disease progression in HIV infection (30, 35,
68 43, 46). Evidence for the detrimental role of chronic IFN-I responses has come from the natural
69 hosts of SIV, such as African green monkeys (AGMs) and sooty mangabeys (SMs) (47). These
70 natural hosts have asymptomatic SIV infections, despite viremia similar to those found in
71 HIV/SIVmac infections. Non-pathogenic SIV infection in natural hosts associates with a lack of
72 chronic inflammation (29). This is despite a robust inflammatory response, exemplified by an
73 increase in ISG expression and production of cytokines, such as IL-15, during acute infection (6,
74 19, 20). However, this inflammatory response is downmodulated by the end of acute infection.
75 Peak IFN-I levels have been found to be lower in the plasma and lymph node cells of SIV-
76 infected AGMs than in MACs, indicating differences in IFN-I production between the two species
77 *in vivo* (9, 19). Nonetheless, exogenous injection of IFN-I at the peak of IFN-I production did not
78 alter the ISG profile observed in AGMs (20). Moreover, AGM and SM plasmacytoid dendritic
79 cells (pDCs) produce high IFN-I levels upon *in vitro* stimulation with synthetic TLR7/9 ligands, SIV
80 or Herpes simplex virus 1 (HSV) (5, 9, 11).

81 pDCs are responsible for the majority of IFN-I production in blood upon *in vitro* stimulation with
82 HIV and HSV (12, 49). In chronically infected HIV patients, pDCs express increased levels of IFN-I
83 mRNA and protein compared to healthy individuals (26, 27). pDCs sense HIV mainly through the

endosomal Toll-like receptor (TLR)7, although a role for TLR9 has not been excluded (4, 28). Blocking endosomal signaling decreased IFN-I levels and IA in treated immunological non-responders (39). In acute SIV infections, pDCs are the main IFN-I-producing cells in lymph nodes (9, 16).

HIV/SIV infections have an impact on pDC distribution, phenotype and function. It has been shown in SIVmac and SIVagm models that activated pDCs are rapidly recruited from the circulation to lymph nodes (7, 11, 20). Simultaneously, precursor pDCs rapidly egress from the bone marrow during acute SIVmac infection (7, 8). Precursor pDCs have a diminished capacity to produce IFN-I upon viral stimulation (8, 33). In line with this, human pDCs were found to produce less IFN-I upon HSV stimulation during late acute HIV infection, i.e. Fiebig stage III-IV (21, 32). This could also be explained by pDCs becoming refractory after HIV encounter (49). However, another study using purified pDCs reported an increased capacity to produce IFN-I upon AT2-inactivated HIV stimulation during post-acute HIV infection (Fiebig stage V-VI) (44). *In vitro*, HIV-1 is contained in an early endosomal compartment of pDCs, preventing pDC maturation and associating with a continued IFN-I producing capacity (31, 37). AGM peripheral blood mononuclear cells (PBMC) and lymph node cells collected during acute SIV infection mounted a stronger IFN-I response upon HSV stimulation than cells from uninfected animals (11). Thus, contradictory data exists on the IFN-I producing capacity of pDCs during acute HIV/SIV infections. The capacity of natural host pDCs to produce IFN-I in response to SIV during acute infection has not been investigated.

In this study, we investigated the effect of SIV infection on AGM pDC function to identify events that could explain the different inflammatory profiles found in asymptomatic SIVagm infection

compared to pathogenic HIV/SIV infections. We assessed the IFN-I producing capacity of pDCs collected during SIVagm infection in response to *in vitro* stimulation with SIV and HSV. Our data suggest that precursor pDCs, with a decreased IFN-I producing capacity, circulate during acute SIVagm infection. We found that low BDCA-2 expression on pDCs could specifically block the sensing of SIV. Furthermore, we discovered that cells other than pDCs increase their capacity to sense virus during acute infection.

Methods

Ethics statement

All animals were housed in the facilities of the Commissariat à l'Energie Atomique (CEA), Fontenay-aux-Roses, France, permit number: A 92-032-02) or of Institut Pasteur (Paris, France, permit number: A 78-100-3). All experimental procedures were conducted in strict accordance with the international European guidelines 2010/63/UE on protection of animals used for experimentation and other scientific purposes (French decree 2013-118). The CEA complies with Standards for Human Care and Use of Laboratory of the Office for Laboratory Animal Welfare (OLAW, USA) under OLAW Assurance number #A5826-01. All animal experimental protocols were approved by the Ethical Committee of Animal Experimentation (CETEA-DSV, IDF, France) (Notification numbers: 10-051b and 12-006).

Animals and sample collection

Twenty-four African green monkeys (*Chlorocebus sabaeus*) from Caribbean origin and ten Chinese rhesus macaques (*Macaca mulatta*) were used. Blood was collected by venipuncture on sodium heparin tubes that were shipped to Institut Pasteur. Fifteen AGMs were infected via

intravenous inoculation with 250 TCID₅₀ of SIVagm.sab92018 and displayed high viremia levels, as described previously (10, 20). For eight AGMs, blood was drawn at days 2, 4, 7, 9, 11, 14, 25, 31, 59, 85, 122, 183, 241 and 354 post infection (p.i.). Three more AGMs were sampled at days 7, 14, 31 and 65 p.i. Another four AGMs were sampled at days 2, 4, 7, 9, 11, 14, 28, 43 and 58 p.i.

Stimulations

PBMCs were isolated from whole blood after 1:1 dilution in PBS (Life Technologies), using Lymphocytes separation medium (Eurobio). Cells were spun down at 1100xg for 10' in 50mL Leucosep tubes (Greiner Bio-one) and buffy coats were collected. Residual red blood cells were lysed for 6' at room temperature in a sterile filtered buffer consisting of 1g/L potassium bicarbonate, 8.3 g/L ammonium chloride (both from Sigma) and 1 mM EDTA pH 8 (Life technologies). Cells were cultured at 0.5x10⁶ cells/well in 24-wells plates (Costar) at 37° C, 5% CO₂ and at 10⁶ cells/mL in RPMI 1640 + glutamax medium (Life technologies) with 10% heat-inactivated FCS (PAA or Eurobio) and penicillin-streptomycin (Life technologies). SIVagm.sab92018 or SIVmac251 was added at a concentration of 1500 ng/mL p27, HSV-1 was added at a 10⁶ TCID₅₀. A151, a gift from Pr. Olivier Schwartz (Institut Pasteur, Paris, France), and G-ODN were added to the cells simultaneously with the virus to block the TLR7 and TLR9 pathway, respectively. Sodium azide was removed from antibodies against IFNαR2 (MMHAR-2, PBL) and BDCA-2 (AC144, Miltenyi) using PD Minitrap G-25 (GE Healthcare Life Sciences), followed by pre-incubation with these antibodies for an hour at 37° C before stimulation with virus. Previously frozen plasma on EDTA from five AGMs per timepoint was pooled for

incubations (four hour at 37° C) with PBMC. PBMC were mock-stimulated in medium for each of the experiments to assess spontaneous production of IFN-I.

Functional Interferon alpha assay

Bioactive Type I interferon was quantified as described earlier (1, 11). In short, Mardin-Darby Bovine Kidney (MDBK) cells were incubated with UV-inactivated supernatants for 18 hours, after which the cytopathic effect of vesicular stomatic virus was determined using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega). IC₅₀ levels were calculated by normalization to a standard using R version 2.15.3 and the “drc” package (40, 41).

Flow cytometry

Antibodies used were: CD3 (SP34-2), HLA-DR (L243), CD123 (7G3), CD45 (D058-1283) (all BD Biosciences), CD20 (2H7, ebioscience), BDCA-2 (AC144) and IFN- α (LT27:295), (both Miltenyi). FcR Blocking Reagent (Miltenyi) was used to block unspecific antibody binding. For intracellular staining, cells were stimulated for two hours, after which brefeldin A was added and cells were incubated for another four hours at 37°C. Then, cells were labeled with surface-binding antibodies for ten minutes at room temperature. After fixation with 4% paraformaldehyde for six minutes at 37°C, cells were permeabilized using 10% saponin and incubated with IFN α antibody for fifteen minutes at room temperature. Events were collected on a LSR-II flow cytometer (BD), running FACS Diva 6.0 software (BD), and analyzed with FlowJo 9.4.10 (TreeStar). Anti-mouse compensation beads (BD Biosciences) were used to define compensation levels.

172

173 **Quantification of relative gene expression**

174 mRNA levels were quantified using RT-PCR as described previously on a 7500 real time PCR
175 machine (Applied Biosystems) (25). Briefly, mRNA was reverse transcribed with the high
176 capacity cDNA reverse transcription kit (Life technologies), followed by qPCR in triplicate with
177 Taqman gene expression assays (Life technologies). The expression of each gene was
178 normalized against that of 18S rRNA and relative expression levels were calculated using the
179 $\Delta\Delta_{ct}$ method.

180

181 **Statistical analyses**

182 Statistical inference analyses were performed using Prism 5.0 (GraphPad). For paired testing of
183 multiple groups without missing data, a Friedman test, followed by Dunn's multiple comparison
184 test, was employed. In case of missing data, the non-parametric Wilcoxon matched-pairs signed
185 rank test (Wilcoxon) was used to test paired observations with no multiple-testing correction.
186 The correlation between two continuous variables was assessed with the non-parametric
187 Spearman test.

188

189 **Results**

190 **SIVagm infection impacts the IFN-I producing capacity during the acute phase**

191 To determine the effect of SIV infection on AGM pDC function *in vivo*, we collected PBMC at
192 different stages before and after infection and stimulated them with SIVagm *in vitro*. We

193 compared IFN-I production to stimulation with another virus, HSV. PBMC were collected at
194 three timepoints before infection to get a stable baseline. To be able to get information on the
195 earliest and later events after infection, cells were collected and stimulated at seven timepoints
196 in acute infection and seven timepoints in chronic infection. Spontaneous IFN-I production by
197 mock-stimulated PBMC was never observed, even during day two and nine p.i., when high IFN-I
198 levels were present in the plasma. The AGM pDC response to *in vitro* SIV stimulation diminished
199 during acute SIV infection starting at day four p.i. (Figure 1A). This diminished response
200 persisted throughout acute infection until day thirty-one p.i. The median IFN-I level upon
201 stimulation with SIVagm before infection was 78 IU/mL, which decreased to 10 IU/mL at the
202 nadir, on days eleven, fourteen and twenty-five p.i. Statistically significant decreases were found
203 at days four and nine p.i. (Wilcoxon, $p = 0.03$ and $p = 0.02$, respectively), compared to the pre-
204 infection values. As pDCs have been reported to be depleted from the blood in acute HIV-1,
205 SIVmac and SIVagm infections (7, 38), we corrected for pDC counts (Figure 1B). Correction for
206 pDC numbers did not change the observed decreased responsiveness to SIV during acute
207 infection. The median of 0.057 IU/mL IFN-I per pDC before infection decreased to 0.022 IU/mL
208 at day four p.i. (Wilcoxon, $p = 0.04$) and 0.014 IU/mL at day nine p.i. (Wilcoxon, $p = 0.008$). The
209 decrease observed in the acute phase was temporary as the median IFN-I level in the chronic
210 phase (after day twenty-five p.i.) returned close to pre-infection levels: 0.044 IU/mL per pDC.

211 Upon *in vitro* stimulation with HSV of PBMC collected in acute SIVagm infection, we observed an
212 increase in the levels of IFN-I produced compared to before infection (Figure 1C). At days two
213 and nine p.i., there was a significant increase in IFN-I production, with levels of 1350 IU/mL
214 (Wilcoxon, $p = 0.02$) and 1200 IU/mL IFN-I (Wilcoxon, $p = 0.008$) respectively, compared to the

median pre-infection level of 239 IU/mL IFN-I. The response to HSV-1 stimulation was not increased at any of the other days. When correcting for pDC numbers, we observed a more sustained increase in IFN-I producing capacity throughout acute and early chronic infection (Figure 1D). The levels of IFN-I produced upon HSV stimulation increased from 0.28 IU/mL per pDC before infection to a zenith of 1.11 IU/mL and 1.12 IU/mL on days nine and thirty-one p.i., respectively. This increase was statistically significant (Wilcoxon, $p < 0.05$) at days seven, nine, fifty-nine and eighty-five. So, while the IFN-I response to SIV was decreased, the responsiveness to HSV was increased during acute SIVagm infection.

Altered capacity to sense virus during acute SIVagm infection

In order to understand the mechanisms underlying this differential response to SIV and HSV, we investigated the hypothesis that the SIV-sensing machinery of AGM pDCs is specifically altered by SIV infection, leading to a decreased capacity to respond to SIV, but not HSV. HIV and HSV are mainly sensed by two distinct receptors in human pDCs, TLR7 and TLR9, respectively (4, 28). We investigated whether the sensing of SIV by AGM pDCs also occurs through endosomal TLRs, by stimulating PBMC of healthy AGMs with SIV in the presence of antagonists to TLR7 (A151) and/or to TLR9 (G-ODN) (Figure 2A). Blocking TLR7 and/or TLR9 decreased the response to SIV by AGM PBMC. IFN-I levels decreased from 60 IU/mL to 3 IU/mL and 10 IU/mL (Wilcoxon, $p = 0.002$ and $p = 0.004$), after blocking TLR7 and TLR9, respectively. Given the stronger than expected inhibition upon TLR9 blockage, we repeated the experiment using MAC PBMC, which were stimulated with either SIVagm or SIVmac. For MAC PBMC, the response to both viruses (median of 147 IU/mL IFN-I) was lost upon blocking TLR7 (median of 14 IU/mL IFN-I), while it was not significantly inhibited upon blocking TLR9 (median of 89 IU/mL, Figure 2A). Since MAC

pDCs reacted the same to TLR9 inhibition irrespective of the viral isolate used, it is unlikely that SIVagm is sensed more broadly than SIVmac. Therefore, AGM pDCs sense SIV through endosomal TLRs as reported for MAC and human before.

BDCA-2 has been reported to be involved in attachment of HIV virions to the pDC surface (34). It has been shown the ratio of precursor pCD expressing low levels of BDCA-2 is increased during acute SIVmac infection (8, 33). If BDCA-2 is involved in the efficacy of endocytosis and sensing, lowered expression would specifically affect SIV but not HSV. We therefore evaluated the expression and function of BDCA-2 with respect to SIV- and HSV sensing. BDCA-2 levels on AGM pDC were lower during acute infection than before infection (mean fluorescent intensity (MFI) of 1486 pre-infection to a MFI of 660 at day 14 p.i., Wilcoxon, $p < 0.01$) (Figure 2B). This lower BDCA-2 MFI in the acute phase probably reflected the circulation of precursor pDCs, expressing low BDCA-2 levels, as BDCA-2 MFI closely correlated with the percentage of CD40⁺ pDCs (Spearman, $r = 0.49$, $p = 0.0001$) and HLA-DR expression on pDCs (Spearman, $r = 0.75$, $p < 0.0001$) (Figure 2C). These molecules are expressed at lower levels on precursor pDC (33). To assess the role of BDCA-2 in SIV sensing by pDCs, we stimulated AGM PBMC with SIV and HSV in the presence of BDCA-2-blocking antibodies (Figure 2D). Blocking BDCA-2 prevented the response to SIVagm, but not to HSV, in a dose-dependent manner. An isotype control did not alter the IFN-I response to either SIV or HSV. At the end of the acute phase, BDCA-2 expression was similar to pre-infection levels, concomitant with the recovery of IFN-I production upon SIV stimulation in the chronic phase of SIVagm infection. Thus, the decreased response to SIV correlated with a decrease in BDCA-2 on pDCs and an increase in the frequency of precursor pDC in blood.

Non pDCs produce IFN-I upon HSV stimulation due to a positive IFN-I feedback loop in vivo

A higher ratio of precursor pDC should impact both SIV and HSV responses. In order to further investigate the contrasting observations of an increased IFN-I response upon HSV stimulation and an increased circulation of precursor pDCs during acute SIVagm infection, PBMC collected during acute SIV infection were stimulated *in vitro* with HSV and analyzed by intracellular flow cytometric staining for pDC-associated IFN α production (Figure 2E). A median of 91% IFN α_2^+ pDCs upon stimulation with HSV was observed in healthy animals. During infection, the percentage of IFN α_2^+ pDCs in response to HSV stimulation fell to a nadir of 65% at day fourteen p.i. After the transition from the acute to the chronic phase of infection, AGM pDCs regained their capacity to produce IFN-I upon HSV stimulation. A non-parametric multiple comparison test showed this was significant (Friedman, $p = 0.001$).

Although a decreased number of pDCs were producing IFN-I, a greater amount of IFN-I was detected in the supernatant of HSV-stimulated cells collected in acute infection (Figure 1C). To better understand this increased IFN-I production upon HSV stimulation, we searched for other cellular sources of IFN-I. After stimulation with HSV, up to three percent of CD123 $^-$ cells, thus non-pDCs, were producing IFN α_2 at days two, seven, eleven and fourteen p.i. (Figure 3A). The majority of these IFN α_2^+ cells were CD4 $^-$ T cells, but other HLA-DR $^+$ cells also produced IFN α_2 (Figure 4). In the absence of stimulation, no IFN-I production was observed at any time before and during infection (Figure 3B).

This substantial non-pDC associated IFN-I production was surprising. A positive association between the levels of IFN-I in the plasma and the amount of IFN-I produced after stimulation

280 with HSV-1 existed during the acute phase (Spearman, $r = 0.58$, $p < 0.001$, Figure 5A and 5B). We
281 therefore hypothesized that IFN-I induced a positive auto-feedback loop. To test this hypothesis,
282 PBMC from healthy AGMs were incubated with plasma collected at various timepoints of
283 SIVagm infection and then stimulated with HSV (Figure 5C). Pre-incubation with plasma
284 collected during acute SIVagm infection increased the IFN-I response to HSV four-fold
285 (Wilcoxon, $p = 0.008$) compared to pre-incubation with plasma from healthy animals. In contrast
286 to acute phase plasma, plasma from animals in the chronic phase of infection did not increase
287 the IFN-I response to HSV. To confirm that IFN-I mediated this increased response to HSV, we
288 blocked the IFN α receptor 2 (IFN α R2) on AGM PBMC, incubated these cells with acute phase
289 plasma and subsequently stimulated with HSV. Blocking with α -IFN α R2, but not with an isotype
290 antibody, prevented the increase in IFN-I production (Wilcoxon, $p = 0.008$). Therefore, IFN-I was
291 required for this increased response to HSV.

292 As IFN α R signaling increased the response to HSV, we studied whether molecules involved in
293 HSV sensing were increased during acute SIV infection. Expression levels of four known HSV
294 sensors (TLR2, TLR3, TLR9 and IFI16) and three molecules associated with IFN-I production (IRF3,
295 IRF5 and IRF7) were measured in PBMC during acute SIVagm infection. Expression levels of
296 TLR3, TLR7, IFI16 and IRF7 were increased during acute SIVagm infection (Figure 6). In contrast
297 expression levels of TLR2, TLR9, IRF3 and IRF5 were not increased, or even decreased, during
298 acute SIVagm infection. TLR3 and IFI16 mRNA levels correlated with the amount of IFN-I
299 produced upon HSV stimulation (Figure 5D, Spearman, $r = 0.37$, $p = 0.01$ and $r = 0.39$, $p = 0.01$,
300 respectively). In contrast, IRF3, IRF5, IRF7, TLR2 and TLR9 expression levels did not associate
301 with the IFN-I response upon HSV stimulation (Figure 5D and not shown). These data show that

SIV infection induces an upregulation of at least two sensors in PBMC via IFN-I, which could cause other cells than pDCs to increase their sensitivity for pathogen-associated molecular patterns and to produce IFN-I upon viral encounter.

Discussion

During acute SIV infection, AGM PBMC produced less IFN-I upon *in vitro* stimulation with SIV, but more upon stimulation with HSV, compared to before infection. Blocking BDCA-2 reduced the *in vitro* IFN-I response to SIV, but not to HSV. During acute infection, we observed a lower BDCA-2 expression on pDCs, which could thus specifically impact SIV sensing. Moreover, our data suggest an egress of precursor pDCs during acute SIVagm infection, similar to what has been reported for SIVmac infection (7, 8). As precursor pDC are less efficient in producing IFN-I (33), this could also explain the decreased response to SIV.

While the IFN-I production increased upon stimulation of PBMC with HSV during the acute phase, the percentage of pDCs that was producing IFN-I in response to HSV decreased. Surprisingly, cells other than pDCs, notably T cells and also HLA-DR+CD3- cells, started producing IFN-I upon *in vitro* stimulation with HSV during acute SIVagm infection. These data are in agreement with immunohistochemistry data from the spleen of late-stage HIV-infected patients where IFN α colocalized only with few pDCs, but rather with other cells, including T and B lymphocytes, mDCs, and macrophages (36). It is possible that in immunocompromised patients, reactivated latent viruses, such as cytomegalovirus, a member of the *herpesviridae* family, were triggering this non-pDC associated IFN α -production, as these are abundant during late-stage HIV infection (14). Moreover, the expanded gut virome and microbial translocation observed in

SIVmac, but not SIVagm, infection could contribute to chronic IA by triggering IFN-I responses in cells normally not associated with these responses (15, 24).

The increased sensing of HSV by non-pDCs was due to a positive auto-feedback loop of IFN-I, which is transiently present at high levels in the plasma during the acute phase of HIV/SIV infections and also in late stage patients. While our data do not exclude that other factors might be required in addition to IFN-I for this enhanced viral sensing, it is unlikely that SIV particles in the plasma induced the increased IFN-I response as plasma from chronically infected animals did not increase responsiveness. The increased response to HSV was associated with increased expression levels of the viral sensors TLR3 and IFI16. These data are in line with a study showing that TLR3-mediated sensing dominates the IFN-I response during *in vivo* HSV infection in mice after the initial pDC-associated IFN-I responses (48). Such increased sensitivity might also occur in HIV and SIVmac infections, which could have gone unnoticed as most studies using viral stimuli other than SIV did not analyze such early and numerous timepoints as done here (11, 21, 32).

Collectively, our data show that SIVagm infection strongly impacts AGM pDC function and dynamics during acute SIV infection, similar to what happens in HIV and SIVmac infections in blood (7, 8). These modifications normalize however after acute SIVagm infection. The previously reported low level of ISG expression during the chronic phase of SIVagm infection is therefore, as suggested earlier, not a reflection of lack of IFN-I producing capacity by AGM pDCs (5, 19). Furthermore, these results suggest that cells that are not classically involved in IFN-I associated inflammation might be contributing to this during chronic HIV infection due to upregulation of viral sensors in response to the existing inflammatory environment. These data

suggest that specifically targeting pDC-driven IFN-I responses is not sufficient to reduce inflammation and that cells other than pDCs could be crucial in maintaining persistent inflammation in HIV infection.

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Conflict of interest

The author(s) declare that they have do not have a commercial or other association that might pose a conflict of interest.

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Figure legends

Figure 1. Dynamics of type I interferon producing capacity during SIVagm infection. We stimulated AGM PBMC collected before and during the course of SIVagm infection and measured IFN-I production. The x-axis displays the day post infection at which cells were isolated and stimulated with (A, B) SIV or (C, D) HSV. The y-axis shows (A, C) the total IFN-I produced after 18 hours of stimulation or (B,D) the same IFN-I production corrected for the number of plasmacytoid dendritic cells among the PBMC. Grey circles represent pre-infection, upward triangles acute infection and downward triangles post-acute and chronic infection timepoints. The dotted line shows the median for all pre-infection timepoint stimulations. Symbols represent individual animals (n = 5-11 for each time point). * Wilcoxon, $p < 0.05$, ** Wilcoxon, $p < 0.01$. The p-values indicate a significant difference compared to the pre-infection baseline.

Figure 2. Molecules involved in SIV sensing by AGM plasmacytoid dendritic cells. (A) PBMC from ten non-infected AGMs (open circles) and seven to nine MACs (closed circles) were stimulated with mock (grey) or SIV_{agm} or SIV_{mac} (black). A151 [5µg/mL] and G-ODN [0.5 µM] were added at the same time as SIV to inhibit the TLR7 and TLR9 pathway respectively. (B) Longitudinal follow-up of MFI levels of BDCA-2 on pDCs during early SIV_{agm} infection. BDCA-2 expression was followed for four AGMs. (C) Expression of BDCA-2, CD40 and HLA-DR was measured simultaneously on AGM pDCs. pDCs were defined as CD20⁻HLA-DR⁺CD123⁺ BDCA2⁺ cells. MFI = mean fluorescent intensity, r = Spearman's correlation. (D) α-BDCA2 or irrelevant IgG1 antibodies were added to PBMC of six uninfected AGM for 1 hour at concentrations of 1 µg/mL and 10 µg/mL. After stimulation with SIV_{agm} (S, light grey) or HSV-1 (H, dark grey) for 18 hours, we measured IFN-I levels in the supernatant. IFN-I levels were normalized to IFN-I produced after no incubation with antibodies. The dotted line represents no difference compared to stimulation without antibodies. (E) Percentage of IFNα₂⁺ pDCs upon *in vitro* HSV stimulation at different timepoints of SIV_{agm} infection. PBMC were collected before and after SIV_{agm} infection and pDCs analyzed for IFNα₂ production after *in vitro* stimulation. pDCs were defined as CD3⁻HLA-DR⁺CD123⁺ cells. Symbols represent individual animals. * Wilcoxon, p < 0.05, ** Wilcoxon, p < 0.01, *** Wilcoxon, p < 0.001

Figure 3. Non pDC-associated IFNα production. Dot plots showing CD123 and IFNα₂ expression on whole PBMC collected throughout SIV_{agm} infection. One representative AGM (SV048) out of four analyzed is shown. (A) Cells after stimulation with HSV. (B) Mock-stimulated cells.

Figure 4. Identification of cell types producing IFN-I upon HSV stimulation. PBMC from one representative AGM (SV061) out of four are shown. The chosen timepoint day two post

infection is representative of days two, nine and eleven post infection. PBMC were gated for distinct cell populations to identify cell types that produce IFN α_2 . 5.5% of the CD4⁺ T cells, 85.7% of the pDCs and 1% of non-T, non-pDC cells were IFN α ⁺ after HSV stimulation. All IFN α -producing cells expressed high levels of HLA-DR.

Figure 5. Auto-feedback loop of IFN-I during acute SIV infection of AGMs. (A) IFN-I levels present in the plasma of eight acutely SIV infected AGMs. (B) Correlation between IFN-I levels in the plasma of AGMs during acute SIVagm infection *in vivo* and the amount of IFN-I produced upon *in vitro* HSV stimulation. (C) Plasma collected from healthy AGMs, acutely infected AGMs and chronically infected AGMs was diluted to 5% in RPMI and added to PBMC of eight healthy AGMs. After four hours of incubation, cells were stimulated with herpes simplex virus 1 (HSV) and after another eighteen hours, supernatants were collected for IFN-I quantification. PBMC were incubated with anti-IFN α Receptor 2 (α -IFN α R2) or with an irrelevant antibody as a negative control at 10 μ g/mL for one hour to block IFN α signaling before adding plasma. Symbols represent individual animals. (D) Correlation between mRNA levels of known HSV sensors in PBMC of AGMs during acute SIVagm infection *in vivo* and the amount of IFN-I produced upon *in vitro* HSV stimulation of the same PBMC. ** Wilcoxon, $p < 0.01$. IFI16 = Interferon, Gamma-Inducible Protein 16. TLR = Toll-like receptor. r = Spearman's correlation.

Figure 6. Gene expression profiles of genes involved in HSV sensing and IFN-I responses. mRNA levels were measured in PBMC from six animals (same animals as in Figure 5) during SIVagm infection. The grey lines indicate individual animals and the black line the median of all animals * Wilcoxon, $p < 0.05$, ** Wilcoxon, $p < 0.01$, *** Wilcoxon, $p < 0.001$. The p-values indicate a significant difference compared to the pre-infection baseline.

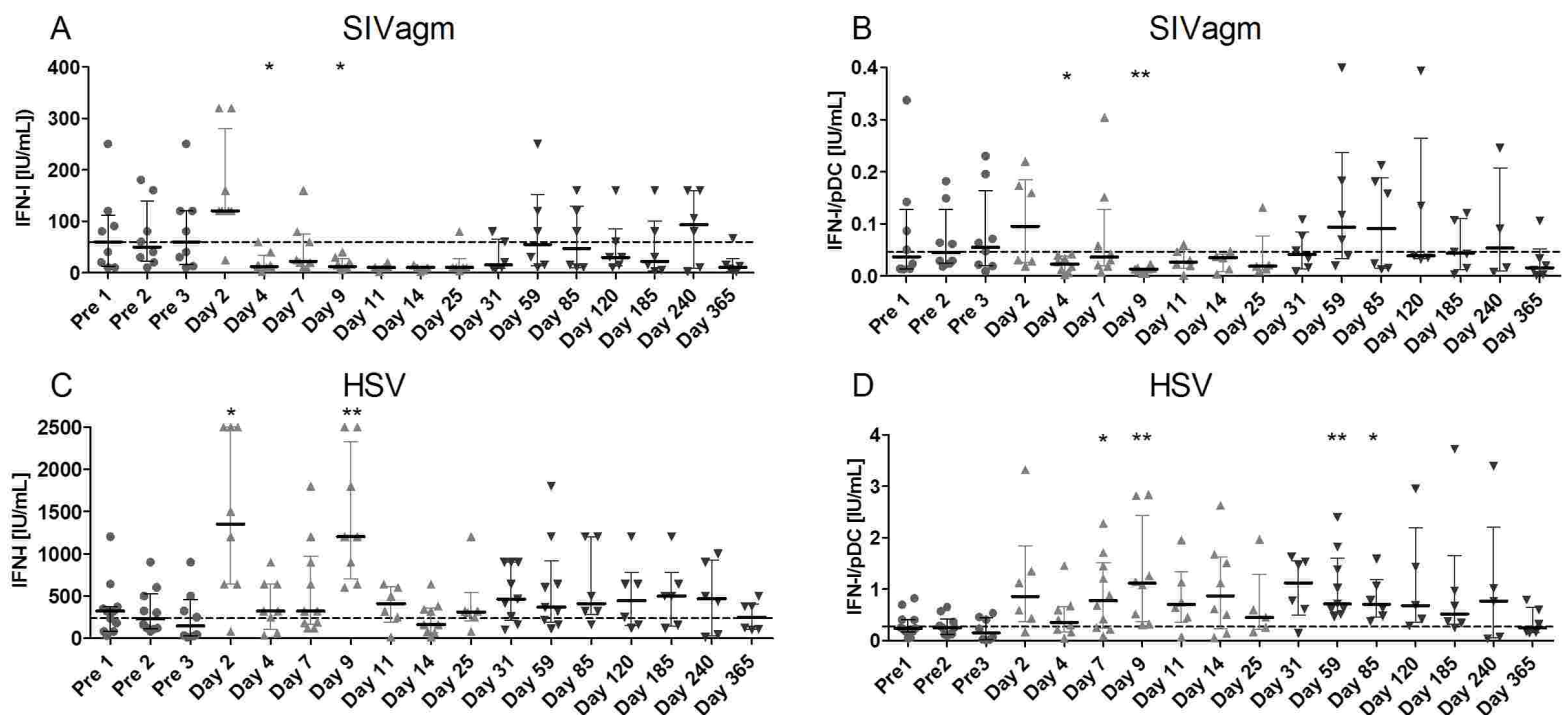


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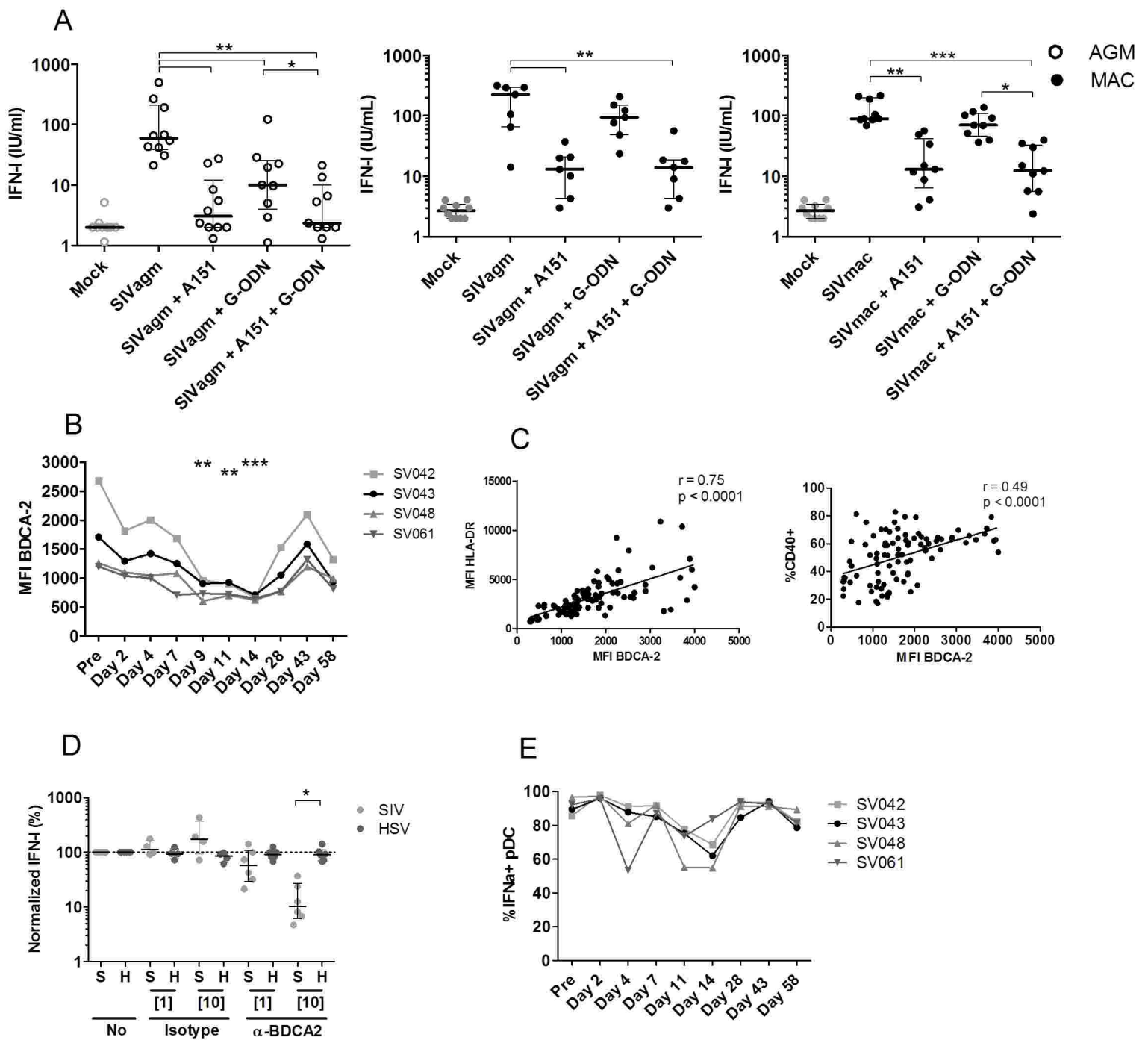


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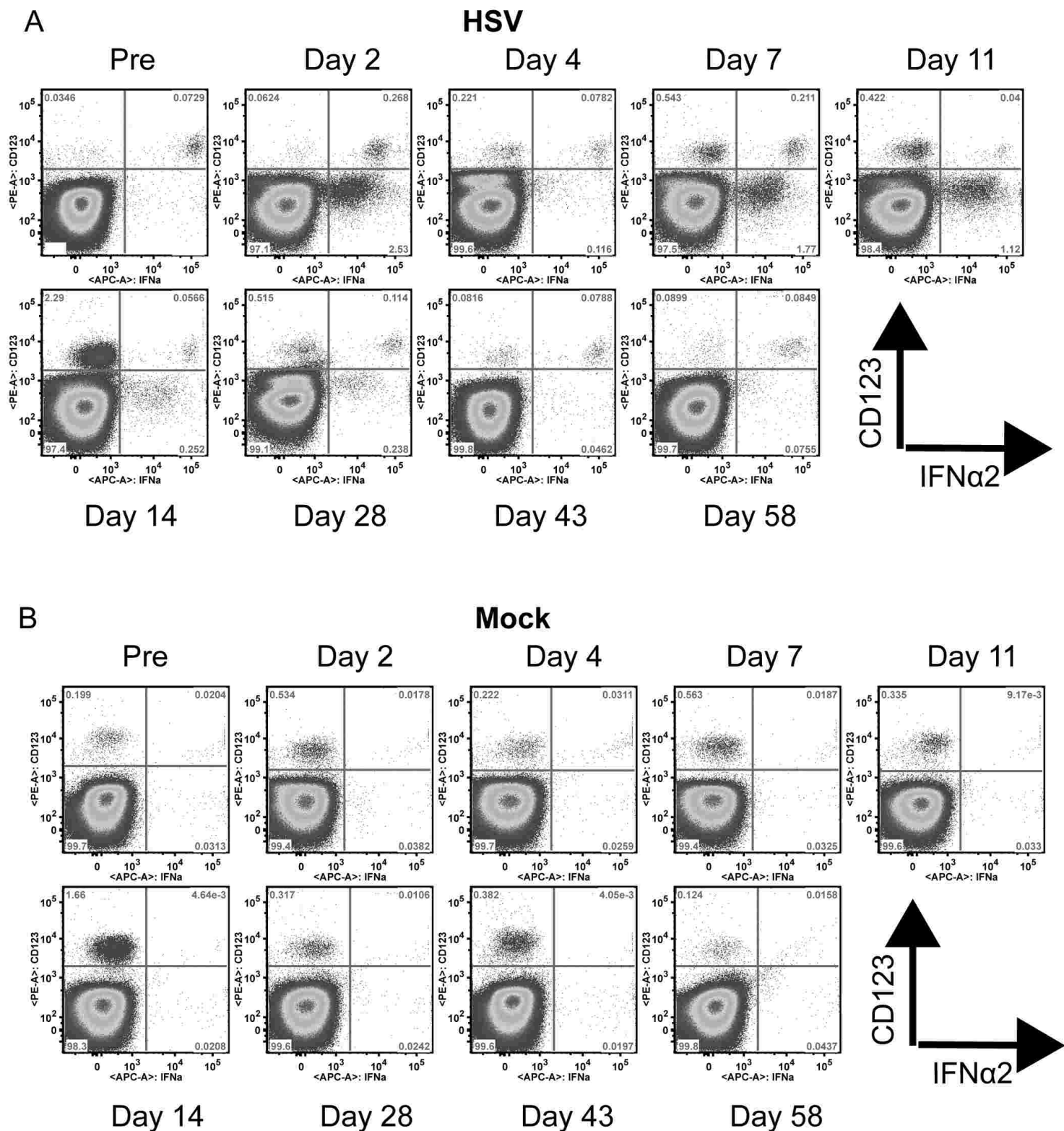


Figure 3. Non pDC-associated IFN α production. Dot plots showing CD123 and IFN α 2 expression on whole PBMC collected throughout SIVagm infection. One representative AGM (SV048) out of four analyzed is shown. (A) Cells after stimulation with HSV. (B) Mock-stimulated cells.

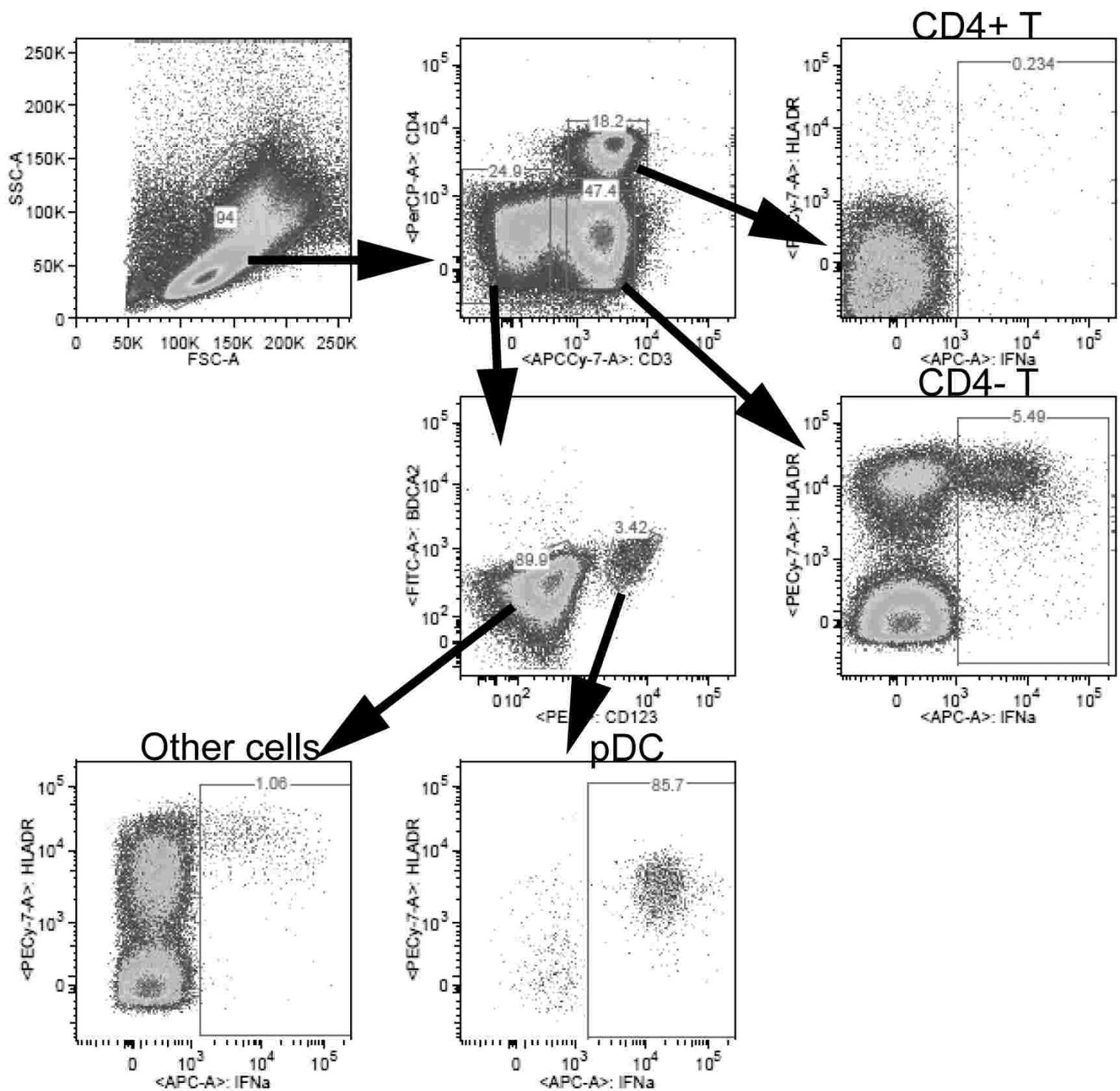


Figure 4. Identification of cell types producing IFN-I upon HSV stimulation. PBMC from one representative AGM (SV061) out of four are shown. The chosen timepoint day two post infection is representative of days two, nine and eleven post infection. PBMC were gated for distinct cell populations to identify cell types that produce IFN α 2. 5.5% of the CD4- T cells, 85.7% of the pDCs and 1% of non-T, non-pDC cells were IFN α + after HSV stimulation. All IFN α -producing cells expressed high levels of HLA-DR.

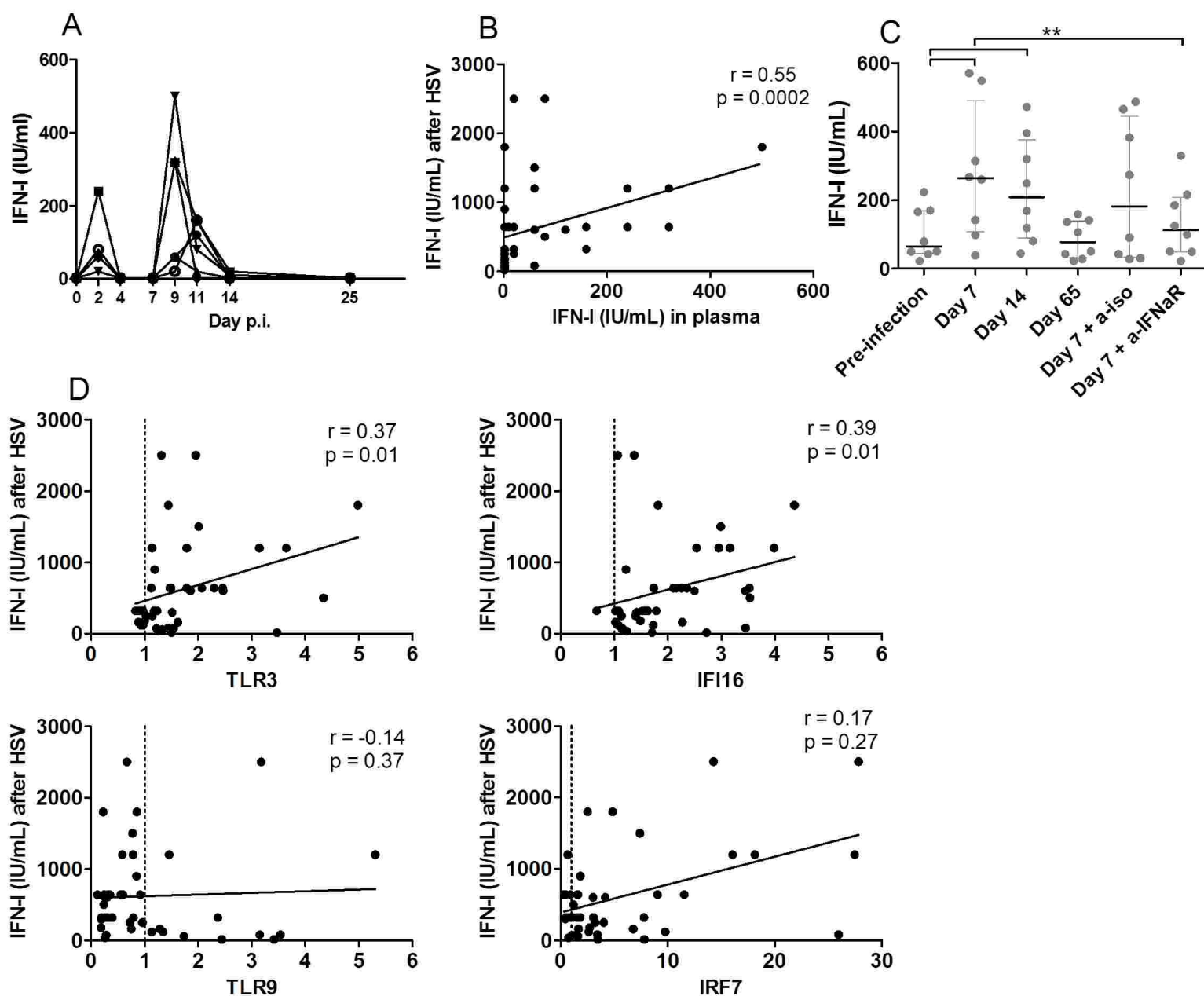


Figure 5. Auto-feedback loop of IFN-I during acute SIV infection of AGMs. (A) IFN-I levels present in the plasma of eight acutely SIV infected AGMs. (B) Correlation between IFN-I levels in the plasma of AGMs during acute SIVagm infection in vivo and the amount of IFN-I produced upon in vitro HSV stimulation. (C) Plasma collected from healthy AGMs, acutely infected AGMs and chronically infected AGMs was diluted to 5% in RPMI and added to PBMC of eight healthy AGMs. After four hours of incubation, cells were stimulated with herpes simplex virus 1 (HSV) and after another eighteen hours, supernatants were collected for IFN-I quantification. PBMC were incubated with anti-IFN γ Receptor 2 (a-IFNaR2) or with an irrelevant antibody as a negative control at 10 ug/mL for one hour to block IFN-I signaling before adding plasma. Symbols represent individual animals. (D) Correlation between mRNA levels of known HSV sensors in PBMC of AGMs during acute SIVagm infection in vivo and the amount of IFN-I produced upon in vitro HSV stimulation of the same PBMC. ** Wilcoxon, $p < 0.01$. IFI16 = Interferon, Gamma-Inducible Protein 16. TLR = Toll-like receptor. r = Spearman's correlation.

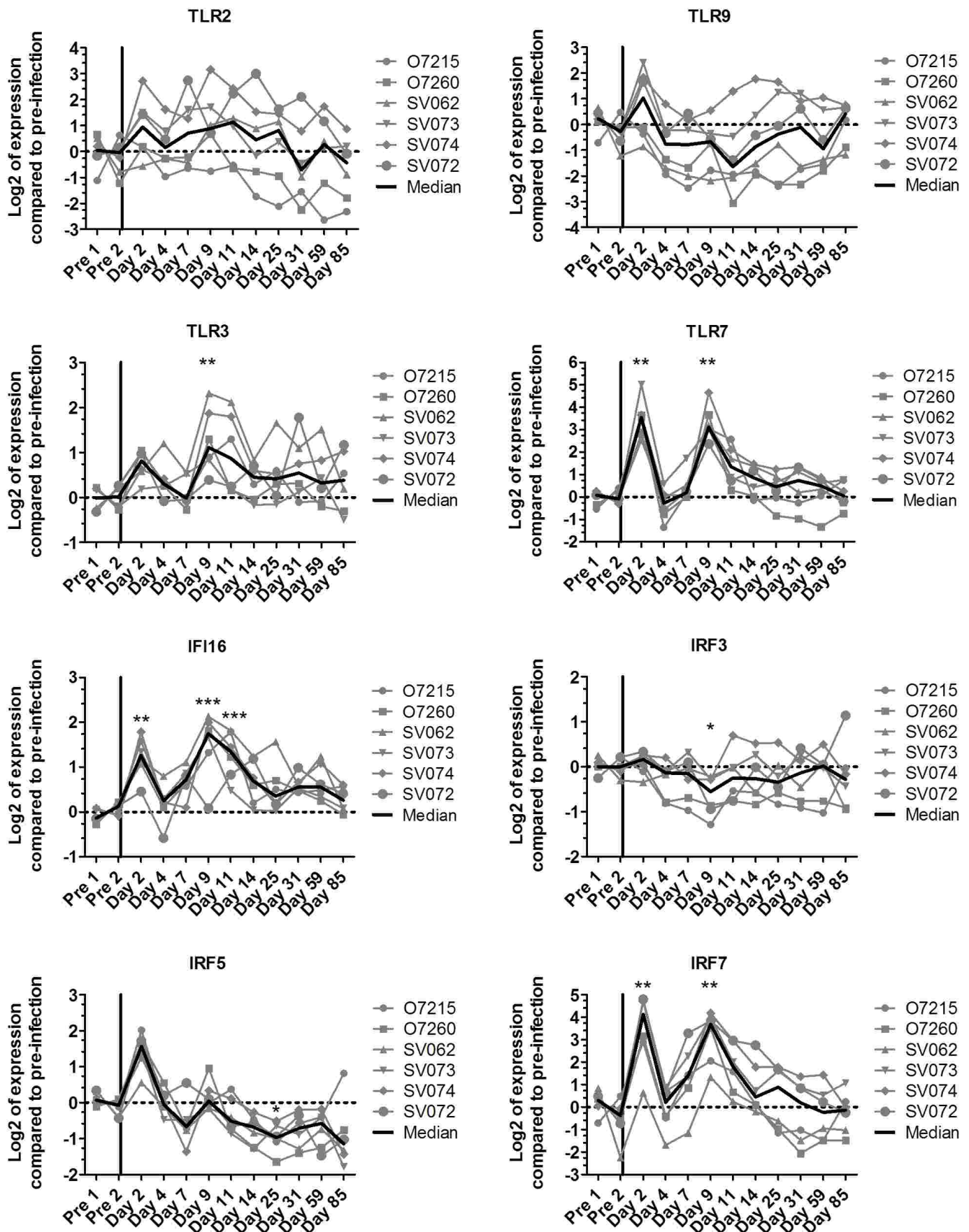


Figure 6. Gene expression profiles of genes involved in HSV sensing and IFN-I responses. mRNA levels were measured in PBMC from six animals (same animals as in Figure 5) during SIVagm infection. The grey lines indicate individual animals and the black line the median of all animals * Wilcoxon, $p < 0.05$, ** Wilcoxon, $p < 0.01$, *** Wilcoxon, $p < 0.001$. The p-values indicate a significant difference compared to the pre-infection baseline.